The Journal of Research on the Lepidoptera

Volume 48: 39-44

THE LEPIDOPTERA RESEARCH FOUNDATION, 31 July 2015

ISSN 0022-4324 (print)
ISSN 2156-5457 (online)

Effect of sperm ejection by females on male fertilization success in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera: Papilionidae)

Nayuta Sasaki[1], Setsuko Todoriki[2] and Mamoru Watanabe[1]*
[1]Graduate School of Life and Environmental Sciences, University of Tsukuba, 305-8572 Ibaraki, Japan. +81-29-853-7282.
[2]National Food Research Institute, National Food Research Institute, NARO, Tsukuba, 305-8642, Ibaraki, Japan
watanabe@kankyo.envr.tsukuba.ac.jp

Abstract. Substantial evidence for cryptic female choice (CFC) has been reported in numerous taxa. However, the mechanisms of CFC are not fully established, partly due to the difficulties in disentangling female from male controls on paternity. The loss of sperm in the female sperm storage organs has been observed in several species of Lepidoptera. Due to the complex morphology of the female genitalia, males may not be able to displace sperm in the spermatheca mechanically. Therefore, the sperm of the male previously mated with might only be ejected by the female. To investigate the effect of sperm ejection by females on male reproductive success, we measured the sperm precedence in the swallowtail butterfly, *Papilio xuthus* (Linnaeus, 1767) using sterilized males. In this species, sperm loss from the spermatheca was more likely to occur in females that received a large spermatophore from the current male. The P_2 value (i.e. the proportion of eggs fertilized by the second male in a double-mating trial) exhibited a bimodal distribution with peaks at 0% and 100%, indicating that most eggs laid after the second mating were sired by just one of a female's mates. When a female had mated twice, the male that transferred the larger spermatophore was more likely to be the principal sire, irrespective of whether he was the female's first or second mate. Therefore, male reproductive success appears to be affected by the ejection of sperm by the female, indicating that female sperm ejection is the mechanism of CFC in this butterfly species.

Keywords: Cryptic female choice; P_2 value; sexual selection; sperm usage; spermatophore.

Introduction

Postcopulatory sexual selection is important for males in polyandrous species, because a male does not always fertilize all the eggs laid by his mates. Hence, male reproductive success does not necessarily increase with the number of matings. Under polyandry males have to compete for fertilization of the eggs even after copulation, a phenomenon termed sperm competition (e.g. Simmons, 2001), which is considered a powerful selective force responsible for shaping male reproductive traits (Harvey & Bradbury, 1994). In addition, female mechanisms that bias paternity toward males with preferred traits, i.e., cryptic female choice (CFC), have also been observed (Thornhill, 1983). Although substantial indirect evidence of CFC has been reported in numerous taxa (e.g. Eberhard, 1991), few studies have conclusively shown paternity bias to be driven by CFC (Fedina, 2007; Pizzari & Birkhead, 2000). Eberhard (1996) suggested that in order to unravel the particular female processes or traits involved in the CFC mechanism, it is necessary to demonstrate that (1) female responses to some conspecific males differ from responses to others, (2) this discrimination between males results in differences in reproductive success of the males, and (3) female biases are associated with particular male characteristics.

Many potential CFC mechanisms such as premature interruption of copulation, lack of sperm transport to storage, the ejection of sperm in storage, lack of ovulation, selective abortion, and others have been proposed (Eberhard, 1996). Of these mechanisms, the loss of sperm in the female sperm storage organ, the spermatheca, has been observed in several species

* *Corresponding author.*

Received: 14 April 2015
Accepted: 26 May 2015

of Lepidoptera (e.g. Pair *et al.*, 1977). Lepidopteran males transfer a single spermatophore containing sperm to the bursa copulatrix of the female during copulation (Fig. 1). Sperm in the spermatophore starts to migrate to the spermatheca several hours after the termination of copulation (Watanabe & Hachisuka, 2005). After this migration, sperm is stored in the spermatheca and later used for fertilization. Because sperm cells in the spermatheca by far outnumber eggs in the female's ovarioles, it has been believed that at least a certain number of sperm in the spermatheca survive throughout the female's lifespan. Etman & Hooper (1979) showed that in the cotton leaf-worm, *Spodoptera litura* (Fabricius, 1775; Noctuidae), sperm in the spermatheca of re-mated females began to decrease in numbers, and the spermatheca became empty just after termination of the second copulation and then increased again. Due to morphological restrictions of the aedeagus of lepidopteran males, they cannot remove rival sperm mechanically from the spermatheca (e.g. Drummond, 1984). In addition, because the loss of sperm from the spermatheca occurred before the sperm migration of the current male started (Xu & Wang, 2010), the sperm in the spermatheca cannot have been washed away by the current male's ejaculate. Thus, females apparently control this process. Using the swallowtail butterfly, *Papilio xuthus* (Linnaeus, 1767), Watanabe & Sasaki (2010) demonstrated that sperm loss from the spermatheca was more likely to occur in females that received a large spermatophore from their second male mate. Therefore, lepidopteran females appear to be able to determine whether or not to eject sperm from a former mate on the basis of some traits of her mates.

If females use sperm loss as a means of cryptic mate choice, presence or absence of sperm loss should result in differences in fertilization success of males. However, because sperm loss is an internal process, it is impossible to observe sperm loss and fertilization success in the same female. So, again using *P. xuthus*, we here compared the P_2 values (i.e., the proportion of eggs fertilized by the second male in a double-mating trial) between females to which larger spermatophores had been transferred from the second than from the first male, and females to which larger spermatophores had been transferred from the first than the second male. If sperm loss has a significant effect on male fertilization success, the P_2 value will be higher in females to which a larger spermatophore has been transferred from the second male, because sperm loss is more likely to occur in females that have received a large spermatophore from the second male.

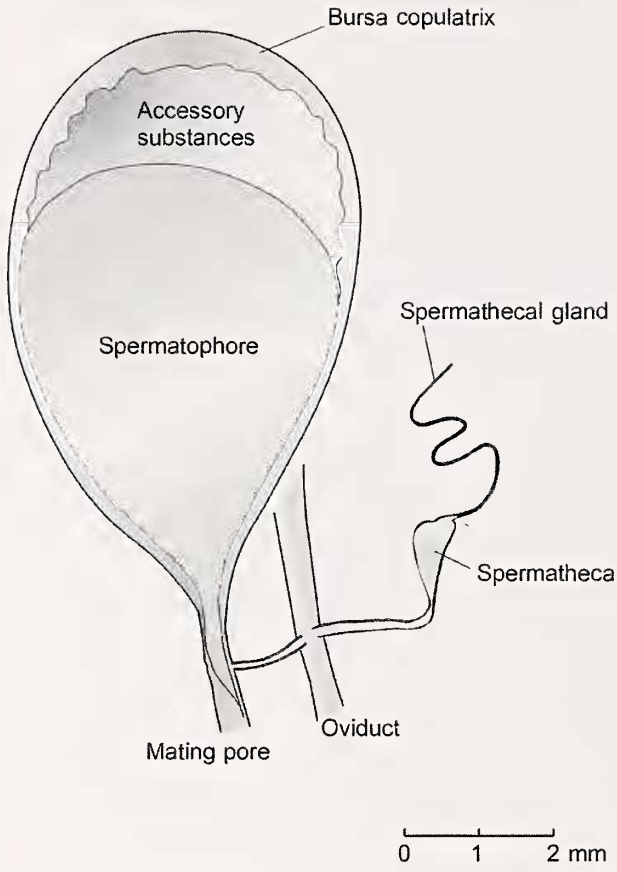

Figure 1. A schematic representation of the *Papilio xuthus* bursa copulatrix, including a spermatophore and accessory gland substances, and the spermatheca in a singly mated female (after Watanabe *et al.* (2000)).

Materials and methods

In the summer of 2010, *Papilio xuthus* females were captured in Ibaraki Prefecture (Japan) to found a captive breeding stock. To avoid diapause, eggs and larvae collected from the females were reared on leaves of the host tree, amur cork, *Phellodendron amurense* Rupr., in the laboratory (room temperature of 28°C and natural long-day photoperiod in July). The laboratory-reared adults were weighed on the day of eclosion (Model AE-240, Mettler, Japan) to an accuracy of ±0.01 mg, and were given individual marks with a felt-tipped pen on their left hind wing. They were kept in net cages (400 × 400 × 450 mm) and fed a 20% sucrose solution for 10 min each day until mating.

The effect of spermatophore size on the pattern of sperm use by females was investigated using females that were mated with a radio-sterilized male and a normal male. We used both virgin and mated males (within 3 days after the first mating) to manipulate the size of

Table 1. Body mass of both sexes and the ejaculate mass for each mating group (mean±SD). Statistical tests refer to ANOVA comparisons across the four sets of experimental animals.

	No. pairs	Female	First male		Second male	
		Body mass (mg)	Body mass (mg)	Ejaculate mass (mg)	Body mass (mg)	Ejaculate mass (mg)
Set N/N	8	450.8±69.5	331.1±42.9	15.5±8.1	340.5±54.3	9.5±7.5
Set S/S	8	448.4±69.3	355.3±50.1	15.1±3.4	304.3±16.2	11.3±5.5
Set N/S	12	396.5±65.6	324.6±53.4	11.8±5.6	322.3±58.3	8.9±6.3
Set S/N	11	446.4±63.4	335.7±58.5	10.6±3.2	339.5±59.6	13.1±5.7
		F=1.58, p=0.21	F=0.55, p=0.65	F=1.97, p=0.14	F=0.92, p=0.44	F=0.99, p=0.41

the spermatophore they were able to transfer to the females. Niihara & Watanabe (2009) showed that a male re-mating within 3 days after a first mating transfer a smaller spermatophore than virgin males. Sterilization of males was carried out by exposure to γ-rays from a ^{60}Co source (*Gamma cell-220*, Nordion International Inc., Kanata, ON, Canada) at a dose of 250 Gy (dose rate: 100 Gy/min). The dose was based on those used in previous studies with other species (Bissoondath & Wiklund, 1997; Seth *et al.*, 2002). Irradiation was performed on the morning of the males' mating day.

Eighty-nine virgin females were hand-paired on the day after eclosion with normal (N) or sterile (S) males. The body mass of the females before and after mating was measured. Due to the impossibility to measure the actual size of the spermatophore without dissection, we used the increase in female mass after mating as a proxy of the size of the spermatophore transferred (±0.01 mg). After the first mating, females were placed individually in egg-laying cages (400 × 400 × 450 mm) with leaves of the larval host tree in order to deposit their eggs and fed 20% sucrose solution for 10 min each day. Leaves were replaced daily, and all eggs deposited were counted.

Three to four days after the first mating, mated females were mated again with either N males or S males. Females were weighed before and after mating to estimate the size of the second spermatophore they had received. After the second mating, females were again placed individually in egg-laying cages with leaves of the larval host tree in order to deposit their eggs and fed 20% sucrose solution for 10 min each day. Leaves were replaced daily until the females died, and all eggs deposited were counted. Probably because of the laboratory conditions such as limited insolation or limited size of the cages, some females failed to lay substantial numbers of eggs. Therefore, only 39 out of 89 females mated twice, which laid more than 10 eggs after the second mating, were considered in the subsequent analyses. The numbers of females mated successively with two N males (set N/N), with two S males (set S/S), first with an N male, followed by a second mating with an S male (set N/S), and first with an S male, followed by a second mating with an N male (set S/N), were 8, 8, 12 and 11, respectively.

The P_2 value was calculated from the viability of eggs (the percentage of eggs that hatched) laid by females in set N/S and set S/N. Because the possibility remains that a certain proportion of eggs fertilized by an N male do not hatch and a certain proportion of eggs fertilized by an S male may hatch, the proportion of eggs fertilized by sperm from the second mating was calculated using the following formula (Sillén-Tullberg 1981):

$$P_2 = 1 - (X_{\text{Set N/S}} - \bar{X}_{\text{Set S/S}})/(\bar{X}_{\text{Set N/N}} - \bar{X}_{\text{Set S/S}})$$

or

$$= (X_{\text{Set S/N}} - \bar{X}_{\text{Set S/S}})/(\bar{X}_{\text{Set N/N}} - \bar{X}_{\text{Set S/S}})$$

where P_2 = the proportion of eggs fertilized by the sperm of the second mate, and $\bar{X}_{\text{Set N/N}}$ and $\bar{X}_{\text{Set S/S}}$ represent the average viability of eggs derived after the second mating from a female mating successively with either two N males or two S males, respectively. $X_{\text{Set S/N}}$ indicates the viability of eggs derived after the second mating from a female mating first with an S male, followed by a second mating with an N male. And $X_{\text{Set N/S}}$ is the viability of eggs derived after the second mating from a female mating first with an N male, followed by a second mating with an S male.

The difference in female body mass, indicating spermatophore mass, among the sets was analyzed using ANOVA. The body mass, indicating spermatophore mass, was also compared between the first and the second males in each set by using the Mann-Whitney *U*-test. P_2 values were analyzed with generalized linear models with binomial errors of the percentage of offspring sired by the second male to mate. As explanatory variables, we used relative ejaculate mass (log(weight gain of females

Table 2. Number of eggs laid (mean±SD) and hatching rate (mean±SE) for each mating group.

	Before the second mating			After the second mating		
	n	No. eggs laid	Hatching rate (%)	n	No. eggs laid	Hatching rate (%)
Set N/N	6	61.3±42.9	82.0±7.1	8	84.4±71.2	86.9±3.5
Set S/S	7	85.3±51.9	22.4±7.3	8	27.3±18.6	17.9±7.6
Set N/S	11	58.5±34.8	75.8±6.2	12	43.8±35.6	66.1±9.0
Set S/N	9	73.0±49.2	12.4±9.4	11	53.2±31.0	57.0±12.6

n: number of females examined

Table 3. Results of the generalized linear model of factors that contribute to variation in P_2 values.

	df	Estimate	SE	z	p
(Intercept)		-0.34	0.92	-0.37	0.715
Relative spermatophore size	1	2.27	1.13	2.00	0.045
Order of sterilized male mate	1	0.57	1.04	0.55	0.582
Number of eggs laid before the second mating	1	0.00	0.01	-0.09	0.929

due to the second mating/that of the first mating)) and the mating order of sterilized males (NS or SN). The number of eggs laid between the first and the second mating was also included as an explanatory variable, to identify the effect of the amount of first male's sperm remaining in the spermatheca on P_2 values. All statistical evaluations were performed with the R (ver. 2.9.1) statistical package (R Development Core Team, 2009). Unless stated otherwise, all values reported are means ± standard deviation.

Results

As shown in Table 1, across the sets of tested individuals the body mass of females did not significantly differ. Likewise, neither the body mass of first nor second mating males differed significantly across the sets (first males: 336.0±51.5 mg, n=39; second males: 327.2±52.1 mg, n=39; U=671.0, p=0.37). Similarly, the ejaculate mass transferred by the first as well as the second males did not significantly differ across the sets (first males: 12.9±5.5 mg, n=39; second males: 10.7±6.2 mg, n=39; U=623.0, p=0.17).

Average viability of eggs laid by females that mated with two N males ($\bar{X}_{\text{Set N/N}}$) was 86.9 %, while that of eggs laid by females that mated with two S males ($\bar{X}_{\text{Set S/S}}$) was 17.9 % (Table 2). By using these values, the P_2 value of each female in the sets N/S and S/N, respectively, was estimated.

P_2 values ranged from 0% to 100%. No individual showed a P_2 value in the range between 30–70% (Fig. 2). Thus, P_2 values showed a bimodal distribution, with either the first or the second male taking "sperm priority." First-male priority (P_2<30%) occurred in 13 of the 23 twice-mated females, and second-male priority (P_2>70%) occurred in the other 10.

The P_2 value was higher when the second male transferred a larger spermatophore than the first one (Fig. 3; z=2.00, p=0.0452). Thus, males who transferred larger spermatophores than their opponents were more likely to take sperm priority. The mating order of the sterilized males did not affect the P_2 value (z=0.55, n.s.) indicating that sterilization per se did not decrease competitive ability of sperm (Table 3). In addition, the P_2 value did not increase with the number of eggs laid before the second mating of females (z=-0.09, n.s.).

Discussion

Watanabe & Sasaki (2010) demonstrated that sperm ejection by female *P. xuthus* occurred when the female received a larger spermatophore from the second male, and they suggested that sperm ejection is the mechanism by which paternity is biased towards preferred males. In the present study, we found that a higher P_2 value was achieved when a female received a larger spermatophore from the second male.

Although we used the weight increase in female mass as a proxy for spermatophore size, there seemed to be a strong relationship between sperm ejection and paternity in this species.

P_2 values of *P. xuthus* exhibited a bimodal distribution with peaks at 0% and 100%. As suggested by Watanabe *et al.* (2000), once sperm ejection occurs, almost all sperm of the first male in the spermatheca is lost, and the remaining sperm is placed in the background of the spermatheca by the second male's apyrene sperm. Consequently, most eggs laid after the second mating will be fertilized by the second male's sperm. On the other hand, when sperm ejection does not occur, little sperm of the second male can enter the spermatheca, because the spermatheca is fully filled by the sperm of the first male. Watanabe & Hachisuka (2005) pointed out that the spermatheca of the lepidopteran female has a restricted storage capacity. LaMunyon (2000) also reported that the storage capacity of the spermatheca of the tobacco budworm, *Heliothis virescens* (Fabricius, 1777), is approximately the mass of one ejaculate. An alternative to the first male sperm priority hypothesis is that females that received a smaller spermatophore from the second male did not allow the second male's sperm to enter the spermatheca. Sperm migration from the spermatophore to the spermatheca depends largely on the female's musculature (Tschudi-Rein & Benz, 1990), and Curril & LaMunyon (2006) suggested that females of the moth *Utetheisa ornatrix* (Linnaeus, 1758), shunt the sperm of unwanted males to different organs rather than to the spermatheca.

The bimodal distribution of P_2 value with peaks of 0% and 100% indicates the low mixing potential of sperm in the spermatheca of female (LaMunyon & Eisner, 1993). Thus, the distribution of P_2 value of *P. xuthus* does not seem not to be explained by numerical sperm competition (but see Harvey & Parker, 2000). The small changes in P_2 values in relation to the number of eggs laid before the second mating of females also suggests that the amount of the first male's sperm in the spermatheca did not affect the fertilization success of both males.

In the present study, females predominantly used sperm from the male who transferred the larger spermatophore for oviposition. The benefits of producing a large spermatophore have been discussed in many lepidopteran species from the male perspective (Gwynne, 2008). The spermatophore represents a male's mating effort, since a large spermatophore can increase the female refractory period (Sugawara, 1979), resulting in more eggs being inseminated by the male's sperm. The spermatophore also functions as a paternal investment, because

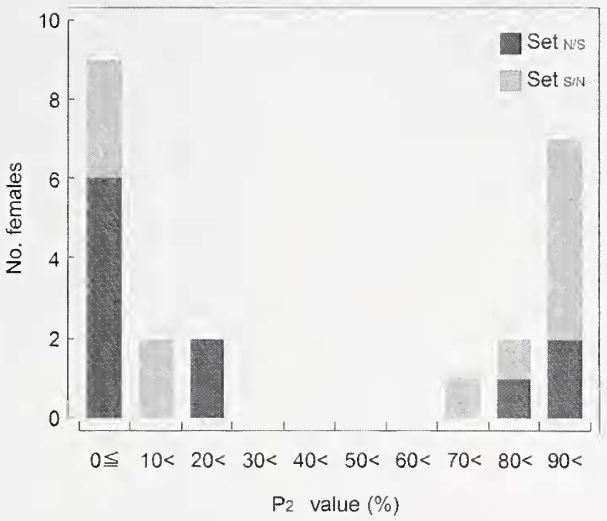

Figure 2. Frequency distribution of P_2 values in 23 twice-mated *Papilio xuthus* females.

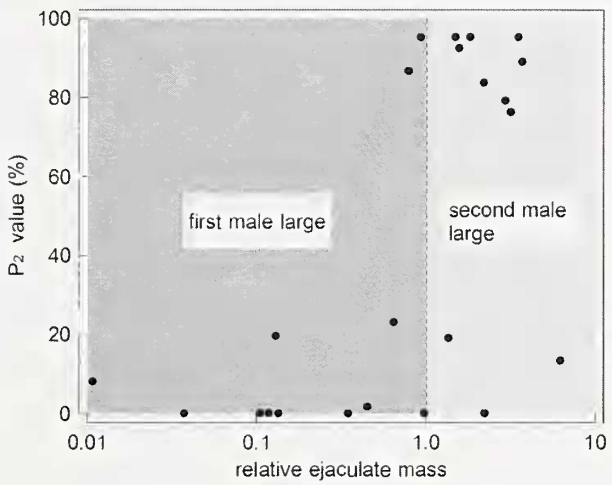

Figure 3. Relationship between the relative ejaculate mass (log(weight gain of females due to the second mating/that of the first mating)) and P_2 values in 23 twice-mated *Papilio xuthus* females. Statistical results are shown in Table 3.

nutrients contained in the spermatophore are used by a female to increase her longevity (Boggs & Watt, 1981) and reproductive output (Watanabe, 1988). Since the male's ability to produce a large spermatophore is heritable (Wedell, 2006), females gain an indirect benefit by choosing sperm from the male that transferred a larger spermatophore.

The bimodal distribution of P_2 values with peaks near 0% and 100% indicate that CFC must have an

impact on male fertilization success in *P. xuthus*. Most eggs will be fertilized by only one male chosen by the female. In addition, ejaculation is costly to the male (Bissoondath & Wiklund, 1997). Males who re-mate within 1–2 days after a previous mating cannot produce as large a spermatophore mass as virgin males (Niihara & Watanabe, 2009). Two to three days of exclusive feeding on nectar are needed for the recovery of the ability to produces a full spermatophore mass (Watanabe & Hirota, 1999). Therefore, males should not re-mate within 1–2 days after a previous mating to avoid selective disadvantages through CFC.

Acknowledgements

We thank Prof. R. Rutowski, Arizona State University, and Dr. T Yokoi, University of Tsukuba, for their critical review of the manuscript. This work was supported in part by JSPS KAKENHI Grant Number 24570019 (MW).

Literature cited

Bissoondath, C. J. & C. Wiklund. 1997. Effect of male body size on sperm precedence in the polyandrous butterfly *Pieris napi* L. (Lepidoptera: Pieridae). Behavioral Ecology 8: 518–523.

Boggs, C. L. & W. B. Watt. 1981. Population structure of pierid butterflies IV. Genetic and physiological investment in offspring by male *Colias*. Oecologia 50: 320–324.

Cook, P. A. & N. Wedell. 1996. Ejaculate dynamics in butterflies: a strategy for maximizing fertilization success? Proceedings of Royal Society of London B 263: 1047–1051.

Curril, I. M. & C. W. LaMunyon. 2006. Sperm storage and arrangement within females of the arctiid moth *Utetheisa ornatrix*. Journal of Insect Physiology 52: 1182–1188.

Drummond, B. A. 1984. Multiple mating and sperm competition in the Lepidoptera, pp.291–370. *In*: Smith R. L. (ed.), Sperm competition and evolution of animal mating systems. Academic Press, London.

Eberhard, W. G. 1991. Copulatory courtship and cryptic female choice in insects. Biological Reviews 66 :1–31.

Eberhard, W. G. 1996. Female control: sexual selection by cryptic female choice. Princeton University Press, Princeton, New Jersey.

Etman, A. A. M. & G. H. S. Hooper. 1979. Sperm precedence of the last mating in *Spodoptera litura*. Annals of the Entomological Society of America 72: 119–120.

Fedina, T. Y. 2007. Cryptic female choice during spermatophore transfer in *Tribolium castaneum* (Coleoptera: Tenebrionidae). Journal of Insect Physiology 53: 93–98.

Gwynne, D. T. 2008. Sexual conflict over nuptial gifts in insects. Annual Review of Entomology 53: 83–101.

Harvey, P. H. & J. W. Bradbury. 1994. Sexual selection, pp. 251–287. *In*: Krebs, J. R. & N. B. Davis (eds.), Behavioural ecology: an evolutionary approach. Blackwell Oxford through Japan UNI Agency Inc., Tokyo.

Harvey, I. F. & G. A. Parker. 2000. 'Sloppy' sperm mixing and intraspecific variation in sperm precedence (P_2) patterns. Proceedings of Royal Society of London B 267: 2537–2542.

LaMunyon, C. W. 2000. Sperm storage by females of the polyandrous noctuid moth *Heliothis virescens*. Animal Behaviour 59: 395–402.

LaMunyon, C. W. & T. Eisner. 1993. Postcopulatory sexual selection in an arctiid moth (*Utetheisa ornatrix*). Proceedings of the National Academy of Sciences of the United States of America 90: 4689–4692.

Niihara, S. & M. Watanabe. 2009. Effects of saline intake until the second mating on the ejaculates and sperm number of male swallowtail butterfly, *Papilio xuthus* Linnaeus. Japanese Journal of Applied Entomology and Zoology 53: 91–96. (in Japanese with English abstract).

Pair, S.D., M. L. Laster & D. F. Martin. 1977. Hybrid sterility of the tobacco budworm: effects of alternate sterile and normal matings on fecundity and fertility. Annals of the Entomological Society of America 70: 952–954.

Pizzari, T. & T. R. Birkhead. 2000. Female feral fowl eject sperm of subdominant males. Nature 405: 787–789.

R Development Core Team. 2009. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org.

Seth, R. K., J. J. Kaur, D. K. Rao & S. E. Reynolds. 2002. Sperm transfer during mating, movement of sperm in the female reproductive tract, and sperm precedence in the common cutworm *Spodoptera litura*. Physiological Entomology, 27: 1–14.

Sillén-Tullberg, B. 1981. Prolonged copulation: a male "postcopulatory" strategy in a promiscuous species, *Lygaeus equestris* (Heteroptera: Lygaeidae). Behavioral Ecology and Sociobiology 9: 283–289.

Simmons, L. W. 2001. Sperm competition and its evolutionary consequences in the insects. Princeton University Press, Princeton, New Jersey.

Simmons, L. W. & M. J. Siva-Jothy. 1998. Sperm competition in insects. pp. 341–434 *In* Birkhead, T. R. & A. P. Møller (eds.), Sperm competition and sexual selection. Academic Press London.

Sugawara, T. 1979. Stretch reception in the bursa copulatrix of the butterfly, *Pieris rapae crucivora*, and its role in behaviour. Journal of Comparative Physiology 130: 191–199.

Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. American Naturalist 122: 765–788.

Tschudi-Rein, K. & G. Benz. 1990. Mechanisms of sperm transfer in female *Pieris brassicae* (Lepidoptera: Pieridae). Annals of the Entomological Society of America 83: 1158–1164.

Watanabe, M. 1988. Multiple matings increase the fecundity of the yellow swallowtail butterfly, *Papilio xuthus* L., in summer generations. Journal of Insect Behavior 1: 17–29.

Watanabe, M., M. Bon'no & A. Hachisuka. 2000. Eupyrene sperm migrates to spermatheca after apyrene sperm in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera: Papilionidae). Journal of Ethology 18: 91–99.

Watanabe, M. & A. Hachisuka. 2005. Dynamics of eupyrene and apyrene sperm storage in ovipositing females of the swallowtail butterfly *Papilio xuthus* (Lepidoptera: Papilionidae). Entomological Science 8: 65–71.

Watanabe, M. & M. Hirota. 1999. Effects of sucrose intake on spermatophore mass produced by male swallowtail butterfly *Papilio xuthus* L. Zoological Science 16: 55–61.

Watanabe, M. & N. Sasaki. 2010. Pattern of sperm storage and migration in the reproductive tract of the swallowtail butterfly *Papilio xuthus*: cryptic female choice after second mating. Physiological Entomology 35: 328–333.

Wedell, N. 2006. Male genotype affects female fitness in a paternally investing species. Evolution 60: 1638–1645.

Xu, J. & Q. Wang. 2010. Mechanisms of last male precedence in a moth: sperm displacement at ejaculation and storage sites. Behavioral Ecology 21: 714–721.